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Bacterial-Derived Uracil as a Modulator of Mucosal Immunity and Gut-Microbe Homeostasis in *Drosophila*

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SUMMARY

All metazoan guts are subjected to immunologically unique conditions in which an efficient antimicrobial system operates to eliminate pathogens while tolerating symbiotic commensal microbiota. However, the molecular mechanisms controlling this process are only partially understood. Here, we show that bacterial-derived uracil acts as a ligand for dual oxidase (DUOX)-dependent reactive oxygen species generation in *Drosophila* gut and that the uracil production in bacteria causes inflammation in the gut. The acute and controlled uracil-induced immune response is required for efficient elimination of bacteria, intestinal cell repair, and host survival during infection of nonresident species. Among resident gut microbiota, uracil production is absent in symbionts, allowing harmonious colonization without DUOX activation, whereas uracil release from opportunistic pathobionts provokes chronic inflammation. These results reveal that bacteria with distinct abilities to activate uracil-induced gut inflammation, in terms of intensity and duration, act as critical factors that determine homeostasis or pathogenesis in gut-microbe interactions.

INTRODUCTION

The gut epithelia of most metazoan organisms harbor complex microbial communities that range from autochthonous bacteria to allochthonous bacteria (Dillon and Dillon, 2004; Ley et al., 2008). Autochthonous bacteria have evolutionarily adapted to the host gut environment and are capable of permanently colonizing the gut, whereas allochthonous bacteria introduced from external environments transiently interact with gut epithelia by passing through the alimentary flowing stream (Savage, 1977). To adapt to these highly diverse microbial populations in the gut, hosts have evolved to modulate the gut-innate immunity to achieve gut-microbe homeostasis by balancing between an efficient immune response to potentially pathogenic allochthonous bacteria and immune tolerance to symbiotic autochthonous bacteria (Artis, 2008; Sansonetti, 2004). However, this is a paradoxical situation from the classical view point of innate immunity because host immune cells should be able to mount an antimicrobial response against any microorganism, regardless of whether it is commensal or pathogenic, by sensing universal microbe-associated molecular patterns (MAMPs) (Beutler, 2004; Lemaitre and Hoffmann, 2007). Several models have been proposed in mammals and *Drosophila* to explain host immune tolerance to symbiotic gut bacteria. These models includes restricted expression and compartmentalization of pattern recognition receptors, multiple mechanisms to down-regulate NF-κB-dependent innate immune signaling, and compartmentalization of gut bacteria by the mucus layer (Hooper, 2009; Lhocine et al., 2008; Paredes et al., 2011; Ryu et al., 2008). However, the molecular mechanism determining how the gut tolerates symbiotic bacteria without mounting inflammation remains to be elucidated.

Drosophila has proven to be a model of choice in terms of understanding evolutionarily conserved mechanisms underlying host-microbe homeostasis in the barrier epithelia (Apidianakis and Rahme, 2011; Bae et al., 2010; Broderick and Lemaitre, 2012; Chambers and Schneider, 2012; Charroux and Royet, 2012). Recent studies have shown that the *Drosophila* gut is able to produce two distinct antimicrobial effector molecules, microbicidal reactive oxygen species (ROS) and antimicrobial peptide (AMP), to control bacteria (Bae et al., 2010; Lemaitre and Hoffmann, 2007). It has been demonstrated that the dual oxidase (DUOX), a member of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family, plays a pivotal role in controlling opportunistic pathogens in the gut by inducing de novo generation of microbicidal ROS (Ha et al., 2005, 2009a, 2009b). Genetic analyses revealed that two DUOX-regulatory

pathways function in efficient pathogen-induced ROS production; the "DUOX-activity pathway" involving Gαq-PLCβ-Ca^{2+}-mediated signaling modulates DUOX enzymatic activity, whereas the "*DUOX*-expression pathway" modulates *DUOX* gene induction through sequential activation of MEKK1-MKK3-p38 MAPK (Ha et al., 2009a, 2009b). Beyond microbicidal effects, DUOX-dependent ROS were also shown to be involved directly or indirectly in the epithelial cell renewal program through activation of intestinal stem cells during gut infection (Buchon et al., 2009a; Lee, 2009). In addition to DUOX-dependent gut immunity, pathogen infection can also activate the immune deficiency (IMD) pathway and subsequent nuclear localization of the Relish, the p105-like NF-κB, which in turn leads to de novo production of AMPs (Lemaitre and Hoffmann, 2007). It is likely that the DUOX-dependent ROS and IMD-dependent AMPs function in a synergistic and/or complementary manner in the gut (Ryu et al., 2006).

The regulation of the intestinal IMD pathway has been extensively studied. Bacterial-derived peptidoglycan acts as the only known MAMP for IMD pathway activation (Leulier et al., 2003; Royet et al., 2011). In the presence of peptidoglycan derived from symbiotic autochthonous microbiota, IMD pathway activation leads to chronic nuclear translocation of Relish, but AMP expression is actively repressed by various negative regulatory molecules including Caudal (Lhocine et al., 2008; Paredes et al., 2011; Ryu et al., 2008). In this model, the IMD pathway is able to maintain low level of activation that enables gut to tolerate commensal bacteria. The Caudal-mediated AMP repression is shown to be required for protection of commensal bacterial community (Ryu et al., 2008). Upon gut infection, additional peptidoglycan derived from high pathogen density can lead to extra-activation of Relish, which in turn induces AMP expression. In contrast to peptidoglycan-induced IMD activation, peptidoglycan is unable to activate DUOX activity (Bae et al., 2010; Ha et al., 2009b). It has been proposed that the bacterial-derived ligand for DUOX activation other than peptidoglycan activates DUOX-regulatory pathways through G-protein-coupled receptor (GPCR) (Bae et al., 2010; Ha et al., 2009b). Despite the presence of extremely efficient DUOX-dependent antimicrobial responses to pathogenic allochthonous bacteria, symbiotic autochthonous bacteria can still colonize the gut without DUOX activation and play their part in maintaining gut-microbe mutualisms (Bae et al., 2010). However, the microbe-derived factors involved in the activation of DUOX-mediated gut immunity have not yet been determined.

In this study, we provide evidence that bacterial-derived uracil acts as a DUOX-activating ligand in the *Drosophila* gut epithelia. We showed that uracil released by allochthonous bacteria induces homeostatic inflammation, which is required for pathogen clearance and host survival. We further found that some pathobionts, resident autochthonous bacterial species having conditionally pathogenic characteristics, release uracil, which chronically activates DUOX-dependent gut immunity leading to pathology reminiscent of inflammatory bowel diseases. In contrast to pathobionts, the absence of uracil in symbiotic resident bacteria allows for peaceful colonization. Our studies illustrate how *Drosophila* mounts DUOX-dependent gut immunity in response to opportunistic pathogens, how commensal bacteria colonize the gut without immune activation, and how certain commensal bacteria initiate chronic inflammation.

RESULTS

Opportunistic Pathogens, but Not Commensal Bacteria, Activate DUOX-Dependent Gut Immunity

To determine if the intensity of the host DUOX-dependent immune response is distinct following different microbial contacts (i.e., symbiotic bacteria or opportunistic pathogen), we examined bacterial-induced microbicidal ROS production in the gut. For the in vivo ROS imaging, a specific and sensitive method for DUOX-dependent ROS detection was used, which was based on a recently developed rhodamine-based sensor, R19S (Chen et al., 2011). R19S is highly specific to DUOX-dependent HOCl and is unable to react with various other ROS (Chen et al., 2011) including hypothiocyanite (Figure S1A available online). *Drosophila* adults were subjected to oral ingestion with *Erwinia carotovora* subsp. *carotovora-15*, a well-known naturally occurring *Drosophila* pathogen (Buchon et al., 2009b). *E. carotovora* is considered to be an opportunistic pathogen in *Drosophila* because it does not harm the normal host but can cause severe lethality when the host's DUOX-dependent gut immunity is impaired (Ha et al., 2005, 2009a, 2009b). As expected, *E. carotovora* was able to induce DUOX-dependent ROS generation at 1–3.5 hr following bacterial ingestion mainly in the anterior midgut region (Figures 1A, S1B, and S1C). Importantly, however, bacterial-induced ROS generation was at a basal level when flies were fed on major symbiotic gut bacteria (such as *Commensalibacter intestini* A911^T, *Acetobacter pomorum*, and *Lactobacillus plantarum*, which account for more than 98% of the total commensal population of our laboratory *Drosophila*; Roh et al., 2008; Ryu et al., 2008) (Figure 1A). Further analyses showed that intestinal ROS generation was specifically induced by diverse ranges of opportunistic pathogens but mostly absent to distinct bacterial species that act as commensal microbiota in *Drosophila* (Figure S1D). This observation suggests that distinction between commensal and pathogenic bacteria in *Drosophila* can be made on the basis of the in vivo DUOX-activating ability of each bacterium. In contrast to live or lysed *E. carotovora*, formalin-fixed dead *E. carotovora* did not efficiently induce ROS generation, suggesting that a molecule secreted from bacteria is responsible for DUOX activation (Figures 1B and S1E). To test the possible existence of bacterial-secreted ligands for DUOX activation, we prepared culture supernatant of different bacterial species. Because we found that complete bacterial growth media, such as Luria-Bertani medium, could also induce intestinal ROS generation, all of the bacteria used in these experiments were grown on minimal growth media, except for *A. pomorum* and *L. plantarum* due to their total absence of growth in the minimal growth media. We found that *E. carotovora* culture supernatants effectively induced ROS generation in a DUOX-dependent manner, whereas *C. intestini* culture supernatants did not (Figure 1C). In contrast to this differential activation of DUOX-dependent gut immunity, culture supernatants of both *E. carotovora* and *C. intestini* led to comparable levels of IMD pathway activation in the systemic immunity as evidenced by the similar induction levels of

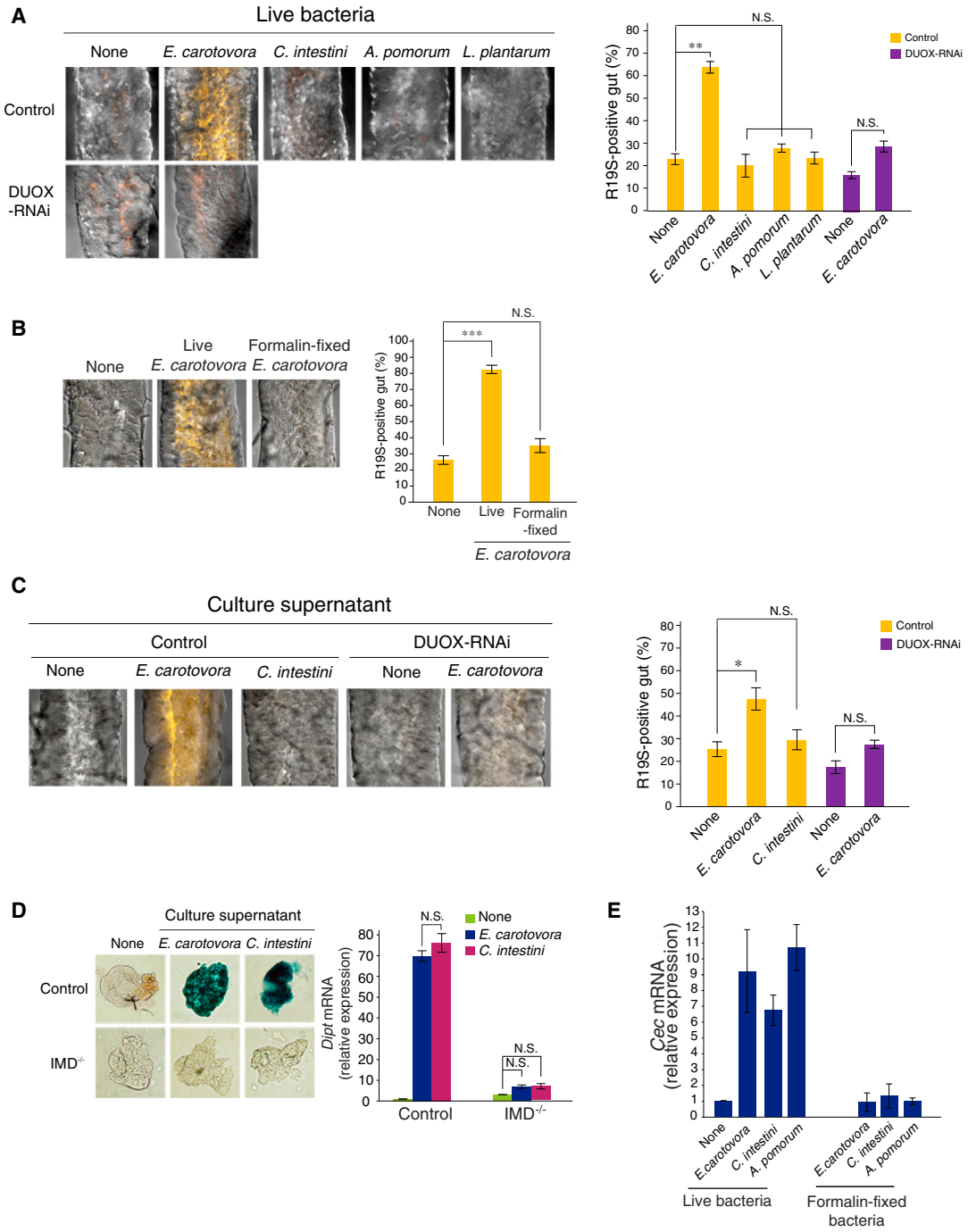


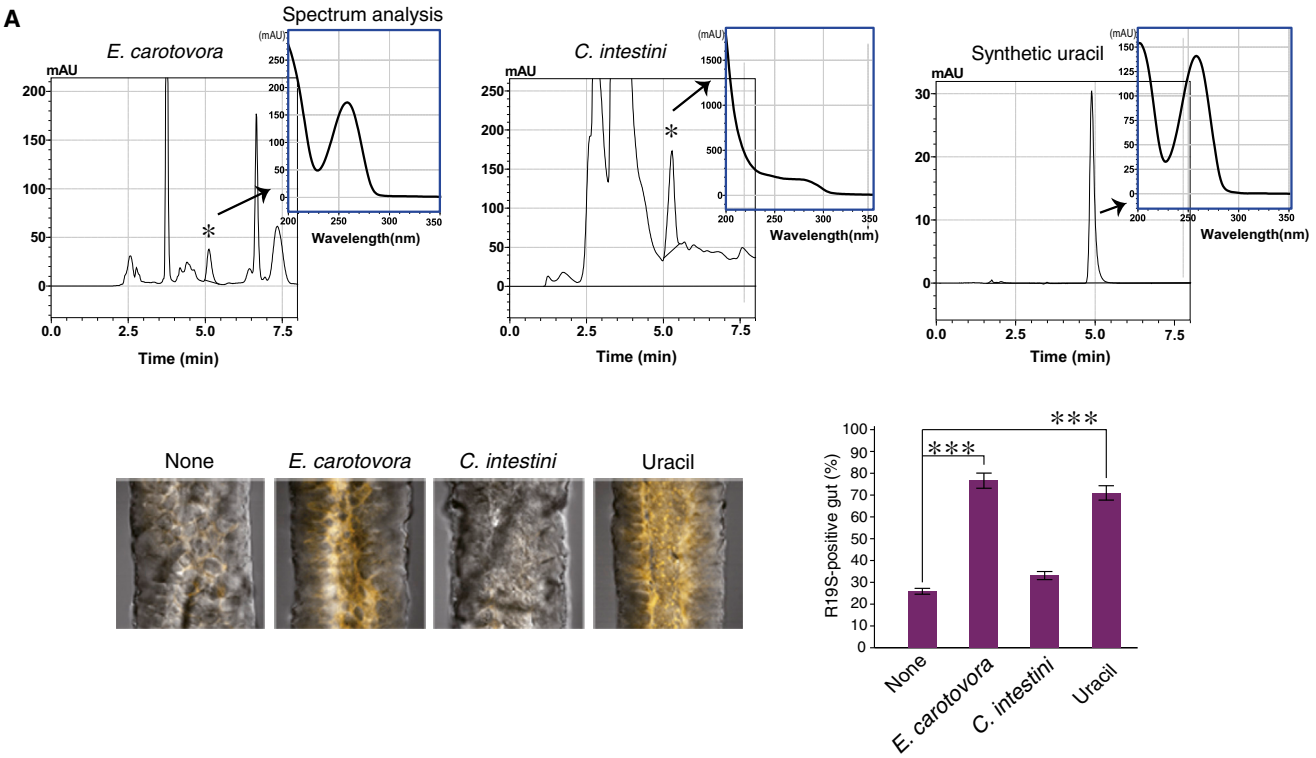
Figure 1. An Opportunistic Pathogen, but Not Commensal Bacteria, Secretes a Ligand Capable of Activating DUOX-Dependent Gut Immunity

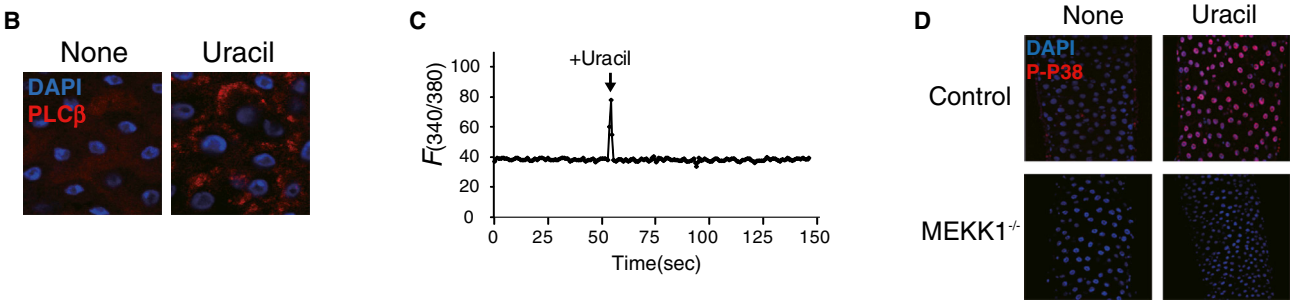
(A–C) DUOX-dependent intestinal ROS generation following ingestion for 1.5 hr with live bacteria (A), formalin-fixed dead bacteria (B), or bacterial culture supernatant (C). ROS production in the anterior midgut was analyzed by HOCl-specific R19S dye. The following fly lines were used: w^{1118} (Control) and *UAS-DUOX-RNAi/+; Da-GAL4* (*DUOX-RNAi*). Data were analyzed using an ANOVA followed by Tamhane's T2 post hoc test; values represent mean ±SEM (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$). N.S. denotes not significant.

(D) Systemic IMD pathway activation. *Dipt* expression was analyzed by quantitative PCR (qPCR) (±SEM) and LacZ staining at 9 hr postinjection. Flies carrying *Dipt-lacZ* (Control) and *Dipt-lacZ; imd*^{1} (*IMD*$^{-/-}$) were used.

(E) Intestinal IMD pathway activation. *Cec* expression was analyzed by qPCR (±SEM) following oral infection with live bacteria or formalin-fixed dead bacteria (~10^{10} bacterial cells) for 9 hr.

See also Figure S1.





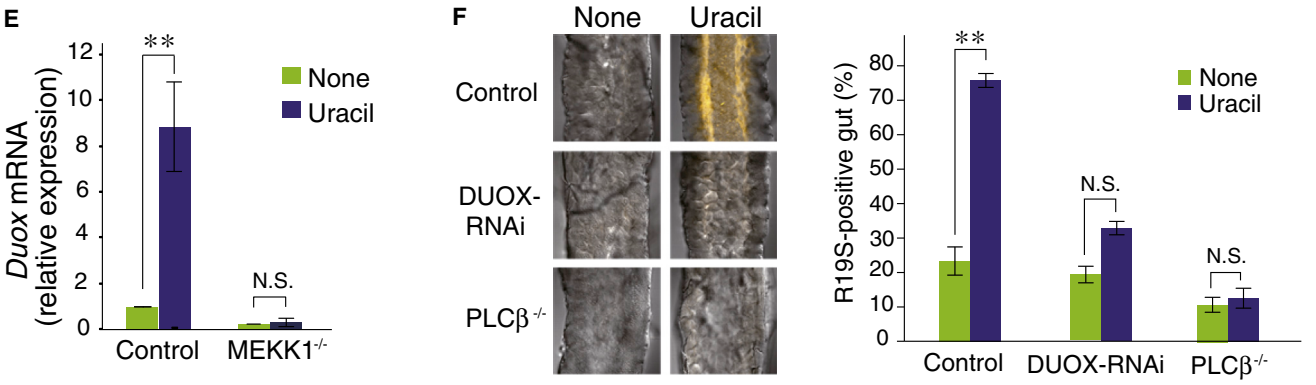


Figure 2. Uracil as a Pathogen-Derived Ligand Capable of Activating DUOX-Dependent Gut Immunity

(A) Purification of *E. carotovora* ROS-inducing factor by HPLC analysis. ROS production in the anterior midgut was analyzed following ingestion of HPLC-purified fractions (1.5 hr). Data are analyzed using an ANOVA followed by Tukey's post hoc test; values represent mean ±SEM (***$p < 0.001$). Peaks of interest (peaks indicated by asterisks) were subjected to spectrum analysis by using a photodiode array detector (blue boxed).

(B) Uracil activates intestinal PLCβ. Transgenic flies carrying *UAS-PLCβ-RFP; Da-GAL4* were used following uracil ingestion (20 nM for 2 hr).

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Relish/NF-κB-dependent AMP gene expression in the fat body (Figure 1D). Furthermore, gut infection experiments with live bacteria, but not formalin-fixed dead bacteria, showed that both Gram-negative pathogens and Gram-negative commensal bacteria induced similar intestinal IMD pathway activation (Figure 1E). These findings indicate that both *E. carotovora* and *C. intestini* secrete similar levels of peptidoglycan, the known IMD-pathway-activating MAMP (Lemaitre and Hoffmann, 2007). Taken together, these findings indicate that two distinct mechanisms, the IMD pathway and the DUOX-activation pathway, are operating concomitantly to mount an immune response against pathogens. As *E. carotovoara*, but not *C. intestini*, is able to induce DUOX activation, *E. carotovora* may secrete an unknown ligand(s) for intestinal DUOX activation.

The *E. carotovora*-Derived DUOX-Activating Ligand Is Bacterial Uracil

To determine the molecular nature of DUOX-activating ligands, *E. carotovora* culture supernatant was purified by reverse-phase high-performance liquid chromatography (HPLC). The DUOX-activating and ROS-inducing ability of HPLC-purified fractions was examined, and a fraction found in the *E. carotovora* culture supernatant was shown to strongly induce in vivo ROS generation in the gut epithelia, whereas a peak with the similar retention time in the *C. intestini* culture supernatants did not show any activity (Figure 2A). This HPLC-purified fraction was subsequently subjected to structure analyses by mass spectrometry and nuclear magnetic resonance (NMR). Chemical analyses revealed that this fraction contained uracil (Figures S2A and S2B). When the HPLC-purified uracil was compared with synthetic uracil by spectrum analysis (Figure 2A) and NMR analysis (Figure S2C), the profiles were indistinguishable, confirming that the pathogen-derived DUOX-activating ligand is uracil. To further investigate whether uracil release occurs in a wide range of bacteria including human pathogens, we performed quantitative analysis of uracil in the supernatant of in vitro cultured bacteria by using liquid chromatography tandem mass spectrometry (LC-MS/MS). As complete medium such as Luria-Bertani medium is found to contain high amounts of uracil (~10 μg/ml), minimal growth media were used to measure the secreted uracil level from the bacteria. Only seven bacterial species were capable of growing in the minimal media among 32 tested bacteria, and their culture supernatants were subjected to the LC-MS/MS analysis. The result showed that pathogens such as *Vibrio fluvialis*, *Klebsiella pneumoniae*, *Shigella sonnei*, *Pseudomonas aeruginosa*, and *Serratia marcescens* secreted significant amounts of uracil (~70–150 ng/10^8 cells) (Figure S2D). Consistent to the above results (Figure 2A), *E. carotovora* secreted high amounts of uracil (~200 ng/10^8 cells), whereas *C. intestini* did not (Figure S2D). Although the uracil amounts released from in vitro cultured bacteria do not necessarily reflect the in vivo uracil release in an epithelial-contacting condition, we could confirm that each bacterium secretes distinct amounts of uracil, a DUOX-activating ligand.

Uracil Is a Specific Agonist for the DUOX-Dependent Gut-Innate Immunity

We have previously proposed that unknown microbe-derived ligands other than peptidoglycan activate the two DUOX-regulatory pathways in *Drosophila* gut epithelia (Ha et al., 2009a, 2009b). Because bacterial-derived uracil is capable of producing intestinal ROS, the effect of uracil on the activation of one or both of these pathways was examined. The result showed that uracil ingestion can activate both the DUOX-activity pathway (PLCβ activation as evidenced by membrane localization of the active form of PLCβ and by Ca^{2+} mobilization) (Figures 2B and 2C) and the *DUOX*-expression pathway (p38 MAPK activation and *DUOX* gene induction) (Figures 2D and 2E). Uracil-induced activation of both DUOX-regulatory pathways led to the generation of large amounts of intestinal ROS in a PLCβ-DUOX signaling-dependent manner (Figure 2F). The presence of commensal bacteria in the gut could not inhibit the ROS-generating ability of uracil, indicating that commensal bacteria are unable to suppress uracil-induced ROS generation (Figure S2E). Coingestion of uracil with an antioxidant chemical, N-acetyl-cysteine, abolished the detection of DUOX-dependent ROS (Figure S2F).

Dose-dependent analysis showed that uracil is capable of inducing intestinal ROS generation from 0.01 nM and most effectively in a range of 1–20 nM (Figure 3A). When we tested the ROS-inducing ability of various uracil-related molecules (other purine and pyrimidine nucleobases as well as eight different pyrimidine analogs including 5-fluorouracil), we found that only uracil is capable of activating DUOX (Figures 3A and 3B), demonstrating the high specificity of uracil in DUOX-dependent ROS generation in *Drosophila* gut epithelia. Furthermore, uracil was also able to activate DUOX-dependent ROS generation in *Caenorhabditis elegans* and human mucosal epithelial cells (Figures S2G–S2I). At present, it is not known how uracil activates PLCβ-induced Ca^{2+} for DUOX-dependent ROS generation. Given that Gαq protein is also required for uracil-induced ROS generation (Figure 3C) and that Gαq-PLCβ-Ca^{2+} pathway acts as one of the main downstream signaling events of GPCRs (Selbie and Hill, 1998), involvement of specific GPCRs responsible for uracil recognition is plausible. In contrast to their abilities to activate the DUOX pathway, uracil was unable to activate the intestinal IMD pathway leading to AMP production (Figure 3D). These results demonstrate that uracil is a specific agonist for the gut-innate immunity capable of inducing DUOX-dependent ROS production but not IMD-dependent AMP production.

(C) Uracil induces intracellular Ca^{2+} in *Drosophila* S2 cells. Uracil (20 nM) was added at a specific time point (indicated by an arrow).
(D and E) Uracil induces p38 activation and *DUOX* expression. The w^{1118} flies (Control) and *MEKK1* mutant flies ($MEKK1^{-/-}$) were used for phospho-p38 analysis following uracil ingestion (20 nM for 2 hr) (D). *DUOX* expression levels were measured by qPCR analysis (±SEM, **$p < 0.005$) at 1 hr postingestion (E).
(F) Uracil induces PLCβ-DUOX-dependent ROS generation. ROS production was examined following uracil ingestion (20 nM for 1.5 hr). The following fly lines were used: w^{1118} (Control), *UAS-DUOX-RNAi/+; Da-GAL4* (*DUOX-RNAi*), and $norpA^7$ ($PLC\beta^{-/-}$). Data were analyzed using an ANOVA followed by Tamhane's T2 post hoc test; values represent mean ±SEM (**$p < 0.005$).
See also Figure S2.

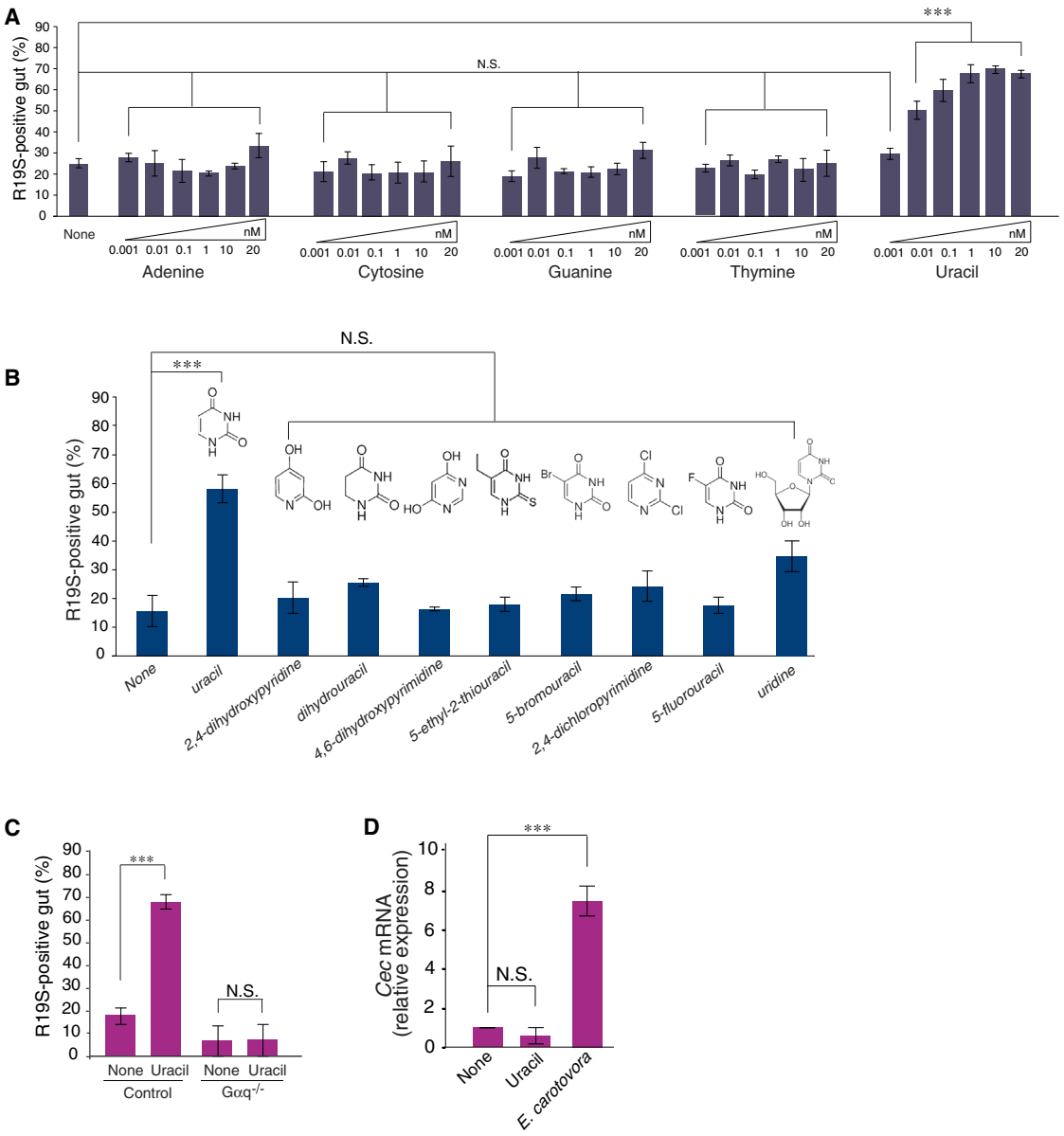

Figure 3. Uracil Is a Specific Agonist for the DUOX-Dependent Gut-Innate Immunity

(A and B) ROS production was examined following ingestion of different nucleobases (A) and pyrimidine analogs (20 nM for each) (B) for 1.5 hr.

(C) Gαq is required for uracil-induced ROS generation. Flies under a w^{1118} (Control) or *Gαq* mutant ($Gαq^{-/-}$) background were used for ROS analysis following uracil ingestion (20 nM for 1.5 hr).

(D) Uracil is unable to activate the intestinal IMD pathway. *Cec* expression was analyzed by qPCR (±SEM; ***p < 0.001) following oral ingestion with uracil (20 nM) or live bacteria (~10^{10} bacterial cells) for 4 hr.

Data in (A)–(C) were analyzed using an ANOVA followed by Tamhane's T2 post hoc test; values represent mean ±SEM (***p < 0.001). See also Figure S2.

The Host Mounts DUOX-Dependent Gut Immunity by Sensing Bacterial-Derived Uracil In Vivo

All of above findings suggest that the gut epithelia selectively mount a DUOX-dependent antimicrobial program against pathogens by sensing pathogen-derived uracil. We reasoned that if this were the case, opportunistic pathogens lacking uracil production would evade DUOX immunity and become virulent to the host. To assess the role of pathogen-released uracil on DUOX activation in an in vivo context, we examined intestinal ROS generation following infection with a uracil auxotrophic mutant (URA^-) pathogen devoid of uracil biosynthesis ability. For this, we performed a transposon Tn*5*-mediated random mutagenesis to generate an *E. carotovora* mutant library and subsequently isolated a single URA^- strain by screening ~6,000 mutant strains (Figure S3A). Sequencing analysis showed that this URA^- strain had a Tn*5* insertion within the

orotate phosphoribosyltransferase gene (*pyrE*, a gene involved in uracil biosynthesis) (Figure S3B). No apparent morphological and/or physiological differences were found between the wild-type (WT) and URA$^-$ strain (Figures S3C–S3F) when these bacteria were cultured in vitro. Furthermore, both WT and URA$^-$ strain induced IMD-dependent systemic and intestinal AMP expression at similar levels (Figures S3G and S3H). Importantly, gut infection experiments showed that this *pyrE* mutant strain (*E. carotovora-pyrE::*Tn*5*), but not other nucleobase mutant strains such as guanine and adenine auxotrophic mutants, had significantly reduced ROS production when compared to the gut infection with parental WT *E. carotovora* strain (Figures 4A, S3I, and S3J). Furthermore, a normal level of infection-induced ROS in the gut was seen following either coingestion of uracil with URA$^-$ strains or ingestion of functionally rescued URA$^-$ strains (i.e., *E. carotovora-pyrE::*Tn*5* strains that ectopically express *pyrE*) (Figure 4A). These results demonstrated that the host mounts DUOX-dependent gut immunity by sensing bacterial-derived uracil in vivo.

Bacterial-Derived Uracil Is Responsible for Stem Cell Modulation and Gut Cell Homeostasis during Gut Infection

Recently, epithelial damage associated with bacterial infection in the gut was shown to accelerate the renewal program of enterocytes through intestinal stem cell (ISC) stimulation, which is essential for gut cell homeostasis and host survival against infection (Buchon et al., 2009a; Chatterjee and Ip, 2009; Cronin et al., 2009; Jiang et al., 2009). Because DUOX-dependent ROS have recently been shown to be involved in controlling ISC turnover during gut infection (Buchon et al., 2009a; Lee, 2009), we determined if uracil is the bacterial-derived ligand that controls the epithelial cell renewal program. To accomplish this, we performed gut infection experiments with WT or URA$^-$ strain and analyzed *escargot*-positive cells using the *escargot-GAL4 > UAS-GFP* system, which allows the identification of ISCs, enteroblasts, and newly synthesized enterocytes (Buchon et al., 2009a; Chatterjee and Ip, 2009; Jiang et al., 2009). The results showed that high numbers of *escargot*-positive cells were present in the midguts of WT bacterial-infected flies in a PLCβ-dependent manner, but that *escargot*-positive cells were significantly reduced in the midguts of URA$^-$ bacterial-infected flies (Figure 4B). Because ISCs are the sole group of dividing cells in the midgut, we further examined the number of dividing cells using an antiphosphorylated histone 3 (PH3) antibody to quantitate the mitotic activity. The results showed that the number of PH3-positive cells was clearly reduced following URA$^-$ pathogen infection when compared to WT pathogen infection (Figures 4C and S4A). When we examined *Su(H)Gbe-lacZ* (a specific marker for enteroblasts) and Prospero (a specific marker for enteroendocrine cells), we found that WT pathogen infection but not URA$^-$ pathogen infection increased the number of enteroblasts in a PLCβ-dependent manner (Figures 4D and S4B). However, no difference in the number of enteroendocrine cells was observed between WT-pathogen-infected gut and URA$^-$-pathogen-infected gut (Figure 4D). Consistent with this impaired epithelial cell renewal process observed in the URA$^-$ pathogen infection, we found more pronounced apoptosis in the URA$^-$-pathogen-infected gut compared to that seen in WT-pathogen-infected gut (Figure S4C). Because the expression of cytokine *Unpaired-3* (*Upd3*) in enterocytes and subsequent activation of Janus kinase signal transducers and activators of transcription (JAK-STAT) signaling in ISCs and enteroblasts are known to be essential for enterocyte differentiation (Buchon et al., 2009a; Cronin et al., 2009; Jiang et al., 2009), we examined whether bacterial-derived uracil is required for Upd3-JAK-STAT signaling activation. Analysis of reporter transgenic flies showed that the number of cells showing *Upd3* expression and subsequent STAT activation was clearly reduced following URA$^-$ pathogen infection when compared to WT pathogen infection (Figure 4E). Importantly, either coingestion of uracil with URA$^-$ strain or ingestion of functionally rescued URA$^-$ strain was sufficient to restore infection-induced Upd3-JAK-STAT signaling activation, ISC proliferation, and differentiation as well as enteroblast accumulation (Figures 4B–4E). Because pathogen-induced Upd3-JAK-STAT pathway activation was abolished in the absence of PLCβ (Figure 4E), we could conclude that the bacterial-derived uracil activates the PLCβ-DUOX-ROS pathway, which in turn acts as an upstream event of the Upd3-JAK-STAT pathway for infection-induced epithelial cell renewal. Furthermore, uracil ingestion alone was sufficient to trigger the Upd3-JAK-STAT pathway activation and epithelial cell renewal process in a PLCβ-dependent manner (Figures S5A–S5E). All of these results together indicate that bacterial-derived uracil is the key factor responsible for bacterial-modulated ISC turnover and the epithelial cell renewal program for gut cell homeostasis during gut infection.

Recognition of Pathogen-Derived Uracil Is Required for Host Survival during Gut Infection

As gut immune responses were virtually abolished in the case of infection with URA$^-$ pathogen, we examined the bacterial persistence and host survival rate following gut infection with these bacteria. When we examined the persistence of these bacteria in the gut, we found that the URA$^-$ pathogen persisted longer than the WT pathogen in the midgut region (Figure 5A). No significant difference in terms of bacterial persistence was observed between WT and URA$^-$ pathogens in the anterior and posterior part of the digestive tract (i.e., crop and hindgut, respectively) (data not shown), suggesting that ingested bacteria are mainly controlled by uracil-modulated gut immunity in the midgut. The prolonged persistence of URA$^-$ pathogen was abolished when the flies were subjected to coingestion of URA$^-$ pathogen with uracil or ingestion of functionally rescued URA$^-$ strain (Figure 5A). Importantly, we found that flies were more susceptible to infection with URA$^-$ pathogen, showing a poor survival rate postinfection, whereas the flies were not adversely affected by infection with WT pathogen or other nucleobase auxotrophic mutants (Figure 5B). Furthermore, we found that coingestion of uracil with URA$^-$ strain or ingestion of a functionally rescued URA$^-$ strain was sufficient to restore the host survival rate (Figure 5B), demonstrating that URA$^-$-pathogen-induced pathology was due to a lack of uracil in the gut lumen. Taken together, these results demonstrate that host survival against gut infection depends on recognition of pathogen-derived uracil and subsequent activation of DUOX-dependent gut immunity.

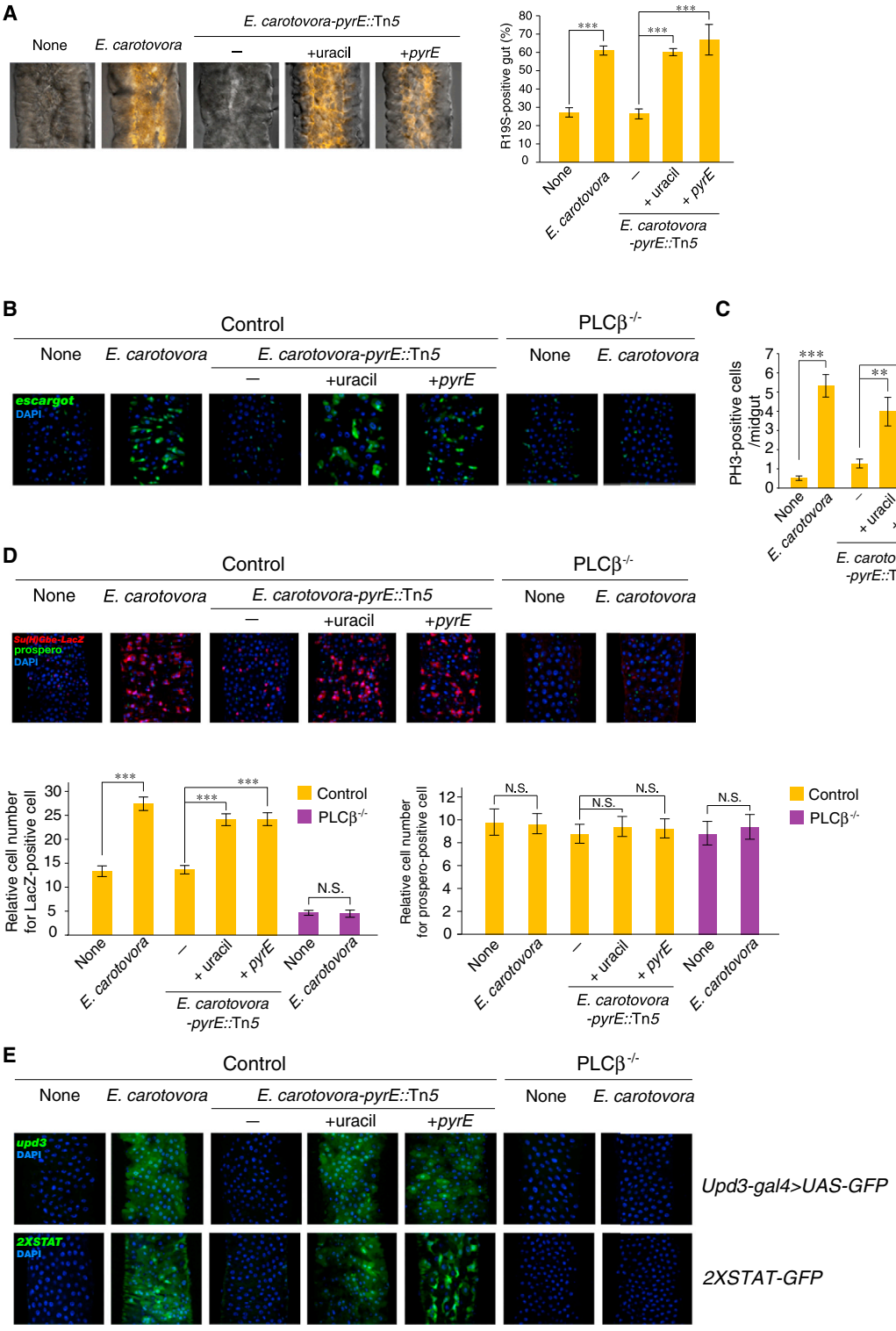

Figure 4. Pathogen-Derived Uracil Is Required for DUOX-Dependent Microbicidal ROS Generation and Intestinal Stem Cell Homeostasis

(A) Reduced ROS production by URA$^-$ pathogen infection. WT pathogen (*E. carotovora*) and URA$^-$ pathogen (*E. carotovora-pyrE*::Tn*5*) were used.

(B–E) Bacterial-derived uracil controls the epithelial cell renewal program. All analyses were performed under a w^{1118} (Control) and a *PLCβ* mutant ($PLC\beta^{-/-}$) background. Analysis of *escargot*-positive cells in the anterior midgut (B) was performed (at 22 hr postingestion) using flies carrying *escargot-GAL4 > UAS-GFP*. Analysis of ISC proliferation by PH3 staining (C) and enteroblast differentiation (D) was performed at 22 hr postingestion. Flies carrying *Su(H)Gbe-LacZ* were used to analyze *Su(H)Gbe-LacZ* and Prospero. The relative cells number of LacZ-positive or Prospero-positive cells was determined by counting the number of

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Chronic Activation of the DUOX-Dependent Gut Immunity by Long-Term Uracil Contact Is Detrimental to the Host

ROS dysregulation has a critical impact on the pathogenesis of many important diseases in mucosal epithelia (Grisham, 1994; Rokutan et al., 2008). Considering the DUOX-activating and ROS-generating ability of bacterial uracil, we hypothesized that constitutive exposure to uracil could produce harmful effects on host physiology. Because it is known that *PLCβ*$^{-/-}$ and *DUOX-RNAi* flies have a short life span under conventional rearing conditions but a normal life span under germ-free conditions (Ha et al., 2009a), germ-free animals were used to examine host physiology following long-term ingestion of uracil. We found an extensive apoptosis in gut cells, which ultimately led to the lethality of control flies in a dose-dependent manner (Figures 5C, 5D, S5F, and S5G). Importantly, this uracil-induced host pathology was completely absent in *PLCβ*$^{-/-}$ or *DUOX-RNAi* flies (Figures 5C, 5D, S5F, and S5G), indicating that uracil induces host cellular damage in a PLCβ-DUOX-dependent manner. Taken together, these findings showed that chronic activation of the PLCβ-DUOX signaling-dependent gut immunity by long-term uracil contact is indeed detrimental to the host due to the excess ROS generation. Because indigenous gut microbiota permanently reside in association with the peritrophic membrane of the midgut epithelia, the above results imply that a gut-dwelling autochthonous bacteria releasing uracil in a constitutive manner may act as a colitogenic factor leading to host pathology.

Chronic Activation of the DUOX-Dependent Gut Immunity due to Long-Term Uracil Release from Autochthonous Bacteria Is the Cause of Pathogenesis

Although some gut symbiotic microbiota are generally beneficial to the host physiology, others can also lead to host pathogenesis in certain circumstances, such as intestinal dysbiosis, a condition whereby an overgrowth of one or more normal species making up the microflora causes intestinal dysfunction and diseases (Garrett et al., 2010; Sokol and Seksik, 2010). Although such abnormalities of microbiota have been associated with many diseases including the inflammatory bowel disease (Garrett et al., 2010; Sokol and Seksik, 2010), the molecular mechanism by which intestinal dysbiosis causes host pathology is poorly understood. In *Drosophila*, it was previously demonstrated that overactivation of IMD-dependent immunity can induce intestinal dysbiosis leading to an overgrowth of *Gluconobacter morbifer* G707^{T}, a minor member of the natural gut microflora (Roh et al., 2008; Ryu et al., 2008). The overgrowth of *G. morbifer* acts as a direct cause of gut pathology and host lethality (Ryu et al., 2008). When the *G. morbifer* population is dominant in the gut (e.g., in the absence of other commensal community members, such as in the case of germ-free animals monoassociated with *G. morbifer*), we observed that *G. morbifer* colonized the midgut region of the intestine (Figure S6A). When we examined the ROS production in *G. morbifer*-monoassociated animals, we found chronic ROS generation accompanying severe gut cell apoptosis in a PLCβ-DUOX signaling-dependent manner (Figures 6A and 6B). We further found that *G. morbifer*-induced ROS were colocalized with colonized bacteria in a PLCβ-DUOX-dependent manner, most intensively in the anterior midgut region (Figure S6B). However, other commensal members such as *A. pomorum*, *C. intestini*, and *L. plantarum* could not induce chronic ROS generation when each of these major bacteria was monoassociated with germ-free animals (Figures 6A, S6B, and S6C). Because the *G. morbifer*-induced chronic DUOX activation is likely the cause of gut pathology, we hypothesized that the constitutive uracil release due to the permanent presence of *G. morbifer* in the gut was the major causal element of *G. morbifer*-induced host pathogenesis. A mutation in the *carbamoyl phosphate synthetase* (*carA*) is known to induce uracil auxotroph (Mergeay et al., 1974). Therefore, we isolated a URA^{-} mutant strain of *G. morbifer* carrying a mutation on the *carA* gene (*G. morbifer-carA::*Tn*5*) using the transposon-based random mutant library by screening ~3,000 mutant strains (Figures S6D and S6E) in order to validate the in vivo role of *G. morbifer*-derived uracil on chronic DUOX activation. Importantly, monoassociation of germ-free flies with *G. morbifer-carA::*Tn*5* strain showed that the this URA^{-} strain did not provoke constitutive ROS generation in the gut (Figure 6C) and led to a significantly reduced gut apoptosis (Figure 6D), which was in contrast to its parental *G. morbifer* strain. When we examined the colony-forming units (CFUs) of colonized bacteria in the guts, we found that the *G. morbifer-carA::*Tn*5* strain colonized gut epithelia as efficiently as the WT *G. morbifer* strain (Figure 6E), confirming that healthy host phenotypes found in the gut of animals monoassociated with *G. morbifer-carA::*Tn*5* were not due to the reduction of colonizing bacterial numbers. When we examined the survival rate of *G. morbifer*-monoassociated flies, we found that the *G. morbifer* strain leads to early host death (Figure 6F), showing its pathogenic nature. Importantly, however, *G. morbifer-carA::*Tn*5*-monoassociated flies showed survival rates that were comparable to those of the control animals (Figure 6F). *G. morbifer*-induced host mortality observed in control flies was abolished in the absence of the PLCβ-DUOX pathway because we observed no difference in the survival rate between *G. morbifer* monoassociation and *G. morbifer-carA::*Tn*5* monoassociation in the case of *DUOX-RNAi* or *PLCβ*$^{-/-}$ animals (Figure 6F), demonstrating that *G. morbifer*-induced disease phenotypes can be effectively reversed by diminishing either bacterial uracil production or host DUOX activity. Furthermore, monoassociation of Gram-positive *L. brevis*, the other minor member of natural gut microflora, with germ-free animals showed that *L. brevis* can also provoke a disease phenotype due to uracil-induced

LacZ-positive or Prospero-positive cells per 100 DAPI-positive cells in multiple images. *Upd3* promoter activity and *STAT* reporter activity (E). Flies carrying *Upd3-gal4* > *UAS-GFP* or *2XSTAT-GFP* were used at 4 hr postingestion.

In the rescue experiment (A–E), coingestion of 1 nM uracil with URA^{-} strain (+uracil) or ingestion with the genetically rescued URA^{-} strain by expressing functional gene (+*pyrE*) was performed. Data in (A), (C), and (D) were analyzed using an ANOVA followed by the Tukey post hoc test; values represent mean ±SEM (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$). See also Figures S3, S4, and S5.

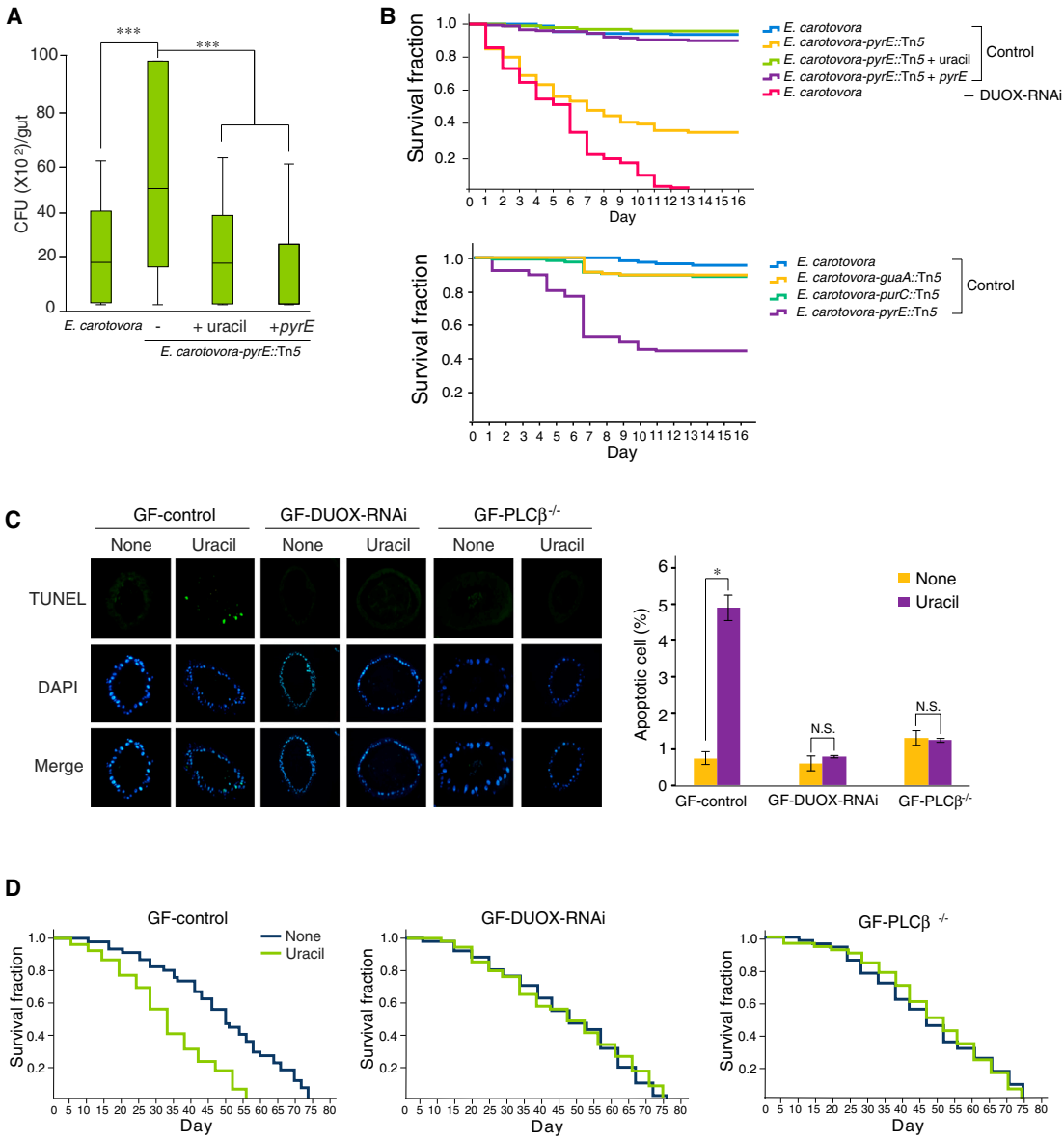

Figure 5. An Appropriate Level of Uracil-Induced Immune Response Is Required for Host Survival

(A) Bacterial persistence in the midgut. CFUs per midgut were analyzed at 6 hr postingestion (n = 30 per each experimental set). Data were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney *U* test using Bonferroni correction to adjust the probability. Bonferroni-adjusted p values were used (***p < 0.001). In box plot diagrams, the black lines and boxes represent the median and first and third quartile values.

(B) URA^{-} pathogen (*E. carotovora-pyrE*::Tn*5*) induces high host mortality compared to that of WT pathogen (*E. carotovora*). *E. carotovora-guaA*::Tn*5* and *E. carotovora-purC*::Tn*5* were also used for guanine and adenine auxotrophic mutants, respectively. The following fly lines were used: w^{1118} (Control) and *UAS-DUOX-RNAi/+; Da-GAL4* (*DUOX-RNAi*). A log-rank analysis on the Kaplan-Meier data (p < 0.001, *E. carotovora* versus *E. carotovora-pyrE*::Tn*5*; *E. carotovora-pyrE*::Tn*5* versus *E. carotovora-pyrE*::Tn*5* + uracil or *E. carotovora-pyrE*::Tn*5* + *pyrE*).

(C and D) Gut cell apoptosis (C) and host death (D) following chronic uracil ingestion (1 nM). Germ-free (GF) animals were used. The apoptosis index (day = 20) was analyzed using an ANOVA followed by Tamhane's T2 post hoc test; values represent mean ±SEM (*p < 0.05). The fly genotypes are described in Figure 2F. A log-rank analysis on the Kaplan-Meier data showed a statistically significant difference in survival between normal food and uracil-containing food in the case of GF-control flies (p < 0.001) but not in the case of GF-*DUOX-RNAi* flies (p = 0.8) or GF-*PLC*$\beta^{-/-}$ flies (p = 0.5).

See also Figure S5.

chronic DUOX activation (Figure S7) similar to that seen in *G. morbier*-monoassociated animals. Taken together, we conclude that the steady release of uracil from two minor commensal bacteria and the consequent chronic activation of PLCβ-DUOX signaling-dependent gut immunity acts as a direct cause of colitogenesis and host mortality.

DISCUSSION

Successful gut-microbe homeostasis is believed to be achieved as a result of a delicate reciprocal interaction between different microorganisms and gut immunity (Artis, 2008; Sansonetti, 2004). The present study demonstrates that uracil rather than conventional MAMPs such as peptidoglycan acts as a microbe-derived factor that modulates DUOX-dependent gut immunity in *Drosophila*. Given that each bacterium likely has a distinct level of uracil release (i.e., URA^+ or URA^- bacteria) as well as a distinct gut-colonizing ability (i.e., allochthonous or autochthonous bacteria), the intensity and duration of DUOX-dependent ROS production in the gut lumen likely varies among four different types of bacterial contacts (Figures 7A–7D). It should be noted that the activation of DUOX-dependent gut immunity is completely dependent on the presence of bacterial uracil, whereas the bacterial gut-colonization ability conferred by innate characteristics of a particular bacterium (i.e., allochthonous or autochthonous bacteria) is a uracil-independent event. To achieve gut-microbe homeostasis, the host should mount adequate DUOX-dependent gut immunity by sensing uracil for transient ROS production and repair of pathogen-induced gut damage to counteract allochthonous bacteria (see Figure 7A for a model). Under this specific immune homeostatic condition, opportunistic *E. carotovora* pathogen could not normally harm host physiology. Because the mutation of a single gene affecting uracil levels in *E. carotovora* is sufficient to produce a gain-of-virulence phenotype leading to host lethality (Figure 5B; see also Figure 7B for a model), uracil recognition is of central importance in *Drosophila* DUOX-dependent gut immunity. In addition, because both DUOX-dependent ROS generation and epithelial cell renewal programs are essential features of gut immunity for host survival during gut infection (Buchon et al., 2009a; Chatterjee and Ip, 2009; Cronin et al., 2009; Ha et al., 2005, 2009a, 2009b; Jiang et al., 2009), the absence of uracil-induced DUOX activation during URA^- pathogen infection resulting in insufficient clearance of URA^- pathogen and impaired repair of pathogen-induced intestinal damage is likely the cause of death.

Immune tolerance to gut-colonizing autochthonous bacteria is essential for long-term cohabitation between host and microbial cells, which remains one of the great enigmas in gut immunity. Excess immune activation to gut microbiota may lead to severe pathogenesis in the mucosa (Bae et al., 2010; Garrett et al., 2010; Ryu et al., 2008; Sokol and Seksik, 2010). We demonstrated that the uracil-deficient nature of major symbiotic gut microbes is highly beneficial, enabling the gut to harbor beneficial microbial cells without immune activation (see Figure 7D for a model). Of note, some conditionally pathogenic autochthonous bacteria (i.e., pathobionts such as *G. morbifer* and *L. brevis*) are normally quiescent but are able to provoke chronic inflammation under dysbiosis conditions (e.g., as in the case of *G. morbifer* dominance in *Caudal* loss-of-function flies [Ryu et al., 2008] or *G. morbifer*-monoassociated gnotobiotic flies used in this study). This situation is reminiscent of inflammatory bowel diseases, wherein some previously commensal resident bacteria cause gut pathology under certain conditions (Andoh et al., 2009; Garrett et al., 2010; Sokol and Seksik, 2010). Our study demonstrated that URA^+ autochthonous bacteria, such as *G. morbifer* and *L. brevis*, can act as colitogenic pathobionts due to their constant uracil release and subsequent chronic DUOX activation (Figures 6 and S7; see also Figure 7C for a model). Deletion of a single microbial gene involved in uracil production (i.e., *G. morbifer-carA::*Tn*5* and *L. brevis*Δ*carA*) is sufficient to induce a phenotypic shift from a colitogenic to commensal type (Figures 6 and S7; see also Figure 7D for a model), suggesting that uracil excretion may be a defining feature of pathogenic versus commensal bacteria. Based on these observations, one can speculate that the shift to uracil production in benign commensal bacteria under certain gut environments may render them to be colitogenic.

One of the fundamental questions is why some bacteria release uracil, whereas others do not. It has been shown for some time that free nucleobases are not normally present intra- or extracellularly in bacteria (Rinas et al., 1995). However, the excretion of free nucleobases, especially uracil and xanthine (but not other nucleobases), can be observed in *Escherichia coli* in response to entry into the stationary phase or in response to the perturbation of balanced growth conditions, such as energy-source downshifts (Rinas et al., 1995). Alternatively, bacterial cell death and lysis under unfavorable growth conditions may release uracil, and if so, the bacterial growth/death rate in an intestinal niche may influence in vivo uracil release. As uracil release is likely influenced by various environmental conditions, such as nutrition availability and cell density, uracil release in a natural niche may be different from that found in laboratory culture conditions. It was proposed that free uracil may originate from the breakdown of stable RNA, such as ribosomal RNA, and that uracil release could be considered as an indicator of a specific metabolic and physiological state of the bacterial community (Rinas et al., 1995). Interestingly, it was found that uracil auxotrophic mutant strains (such as *carA* and *pyrE* mutants) of *Pseudomonas aeruginosa* showed decreased biofilm formation and quorum-sensing phenotypes, which can be restored by exogenous addition of free uracil (Ueda et al., 2009). Such uracil-modulated bacterial responses are known to enhance bacterial virulence that is potentially threatening to host fitness (Attila et al., 2009). It was hypothesized that uracil may act as a bacterial secondary messenger involved in cellular fitness to survive under different environmental conditions (Ueda et al., 2009). Although the exact physiological significance of uracil release in bacteria remains to be elucidated, it is tempting to speculate that *Drosophila* gut epithelial cells have evolved to perceive a change in the bacterial metabolic state through uracil recognition and subsequently mount uracil-induced immunity to efficiently antagonize bacteria before acquiring the resistance and/or virulence mechanism. In this context, symbiotic autochthonous bacteria may have evolved to abolish and/or reduce uracil release in vivo, possibly by modifying the mechanism of uracil excretion, thereby adapting to an intestinal niche. Further in-depth investigation of the detailed bacterial mechanisms controlling uracil release will be needed to better understand the differential regulation of uracil release between commensal and pathogenic bacteria.

Profiling of commensal community members of *Drosophila* showed that different bacterial members (mostly belonging to a subset of bacterial families, such as *Acetobacteraceae*,

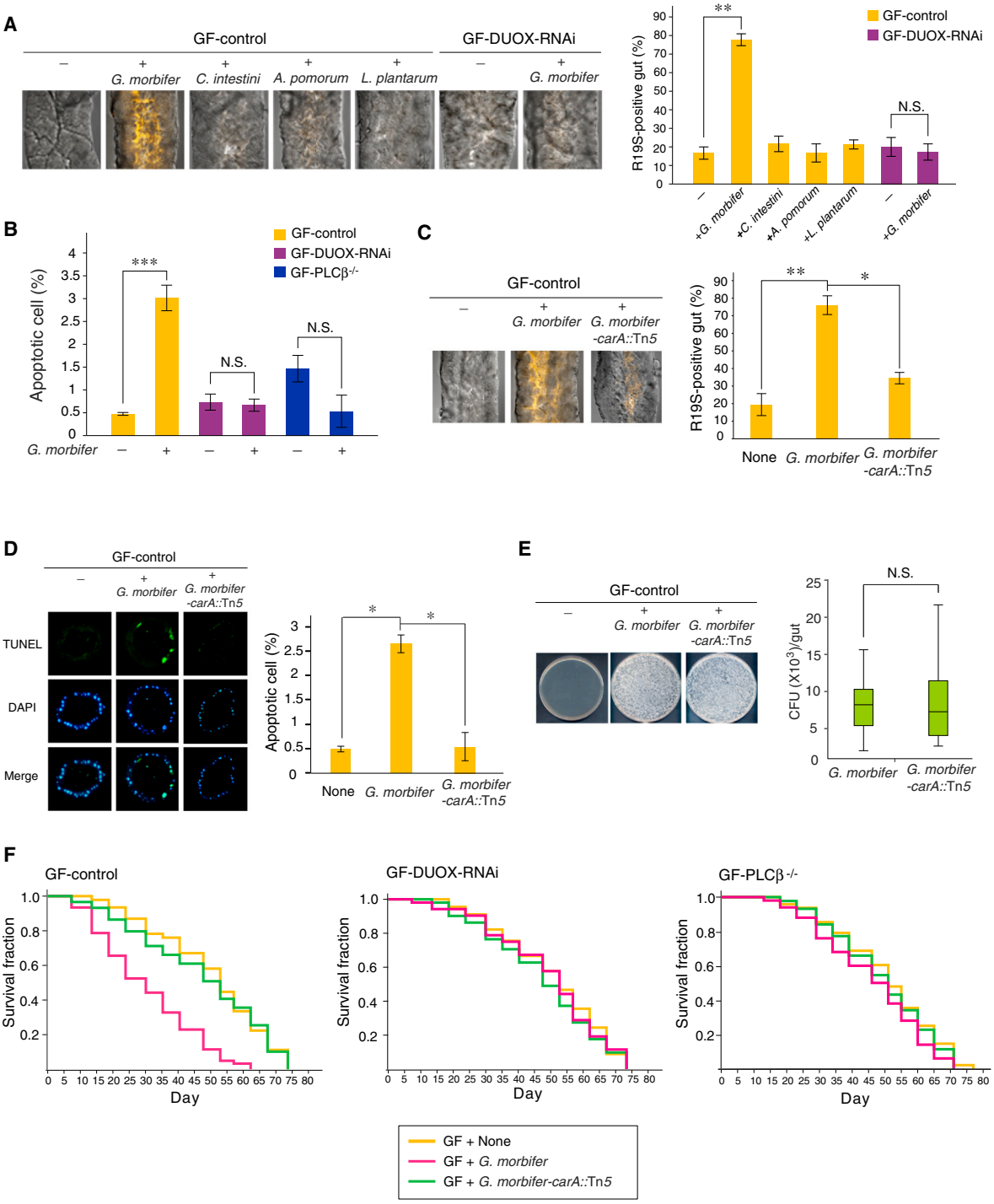

Figure 6. Gut-Dwelling *G. morbifer* Can Act as a Colitogenic Flora due to its Constant Uracil Release and Subsequent Chronic DUOX Activation

(A and B) ROS generation and gut cell apoptosis by *G. morbifer*. The fly genotypes are described in Figure 2F. Basal levels of intestinal ROS were measured in GF animals (GF-control and GF-*DUOX-RNAi*) monoassociated with each of commensal bacteria (A). The apoptosis index (B) is shown.

(C and D) ROS production and gut cell apoptosis are abolished in GF-control animals monoassociated with the URA^{-} mutant strain of *G. morbifer*. Basal levels of intestinal ROS (C) and the apoptosis index (D) were determined.

(E) The URA^{-} mutant strain of *G. morbifer* colonizes gut epithelia as efficiently as WT *G. morbifer*. CFUs were determined using midguts (n = 15) from GF-control animals monoassociated with *G. morbifer* or *G. morbifer-carA*::Tn*5* (day = 13). Data were analyzed using the Mann-Whitney *U* test. In box plot diagrams, the black lines and boxes represent the median and first and third quartile values.

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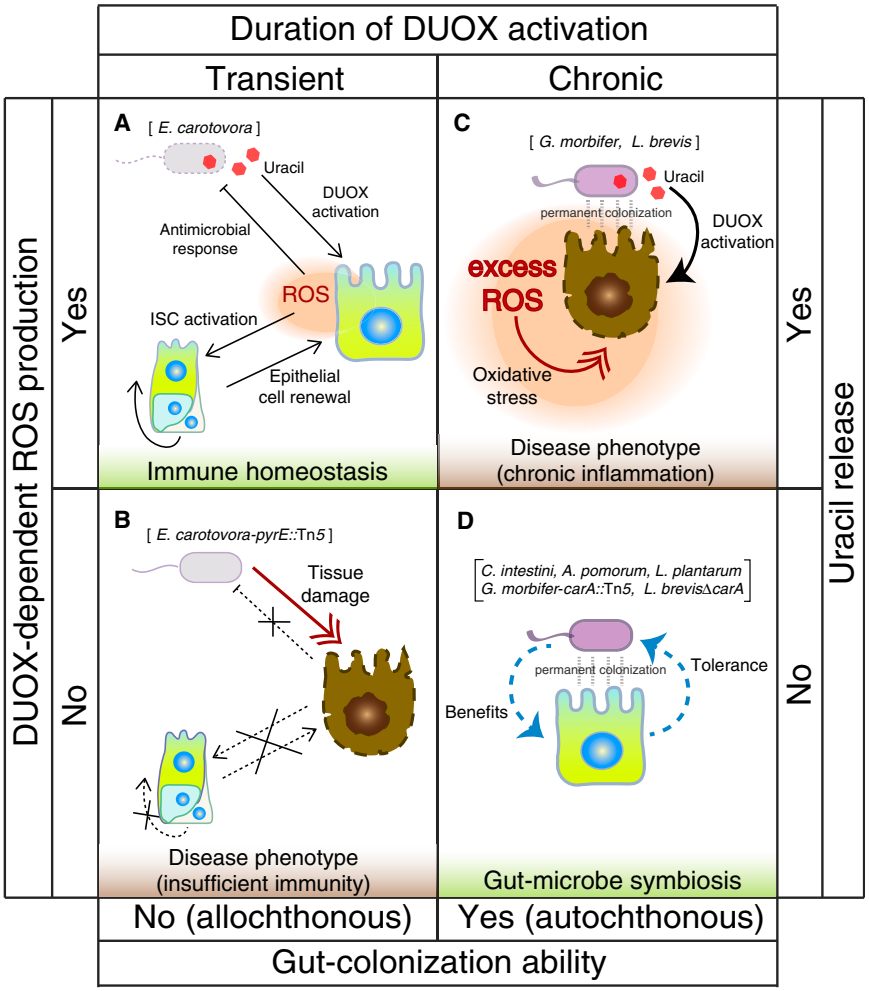

Figure 7. Model of Uracil-Modulated Gut-Microbe Symbiosis and Gut-Microbe Pathogenesis

(A–D) Four different types of bacterial contacts are shown. (A) Regulated activation of gut immunity in response to URA^{+} allochthonous bacterial contacts. (B) Insufficient activation of gut immunity in response to URA^{-} allochthonous bacterial contacts. (C) Chronic activation of gut immunity in response to URA^{+} autochthonous bacterial contacts. (D) Immune tolerance and gut-microbe symbiosis in response to URA^{-} autochthonous bacterial contacts. See Discussion for more details.

Lactobacillaceae, *Enterococcaceae*, and *Enterobacteriaceae*) with high taxonomic diversity at the species level were observed among different laboratory and natural *Drosophila* populations (Chandler et al., 2011; Corby-Harris et al., 2007; Cox and Gilmore, 2007; Ren et al., 2007; Ryu et al., 2008; Storelli et al., 2011; Wong et al., 2011). At present, the exact autochthonous bacterial strains acting as symbiotic/commensal bacteria or pathobionts (such as *G. morbifer* and *L. brevis* in this study) in the *Drosophila* gut is largely unknown. Considering the essential role of bacterial-derived uracil as a modulator of gut-microbe homeostasis, further investigation of the uracil-releasing ability, DUOX-activating ability, and gut-colonizing ability of each bacterial member in a given *Drosophila* gut environment will be required to better understand the physiological characteristics (e.g., autochthonous or allochthonous bacterium, as well as symbiont or pathobiont) of each bacterium in the gut.

Recently, *C. elegans* DUOX (Ce-DUOX) was also shown to be required for pathogen-induced ROS production as a protective host-innate immune mechanism (Hoeven et al., 2011), suggesting the possible existence of a common ligand for DUOX activation in response to pathogens. In mammals, DUOX is known to be expressed in microbe-contacting barrier epithelia such as the bronchial and intestinal epithelia (El Hassani et al., 2005; Geiszt et al., 2003). Although it remains to be validated in an in vivo mammalian animal model, there is accumulating evidence suggesting that mammalian DUOX plays an important role in mucosal immunity (Moskwa et al., 2007; Rada and Leto, 2008). Because our preliminary data showed that uracil was also able to activate DUOX-dependent ROS generation in *C. elegans* and human mucosal epithelial cells, uracil-induced DUOX activation seems to be conserved in metazoan epithelial cells. Although a much more complex environment, it would be interesting to investigate whether a similar mechanism is involved in inflammatory pathologies in the mammalian gut.

Given that many mucosal inflammatory diseases arise from abnormal mucosa-microbe interactions (Artis, 2008; Garrett et al., 2010; Sansonetti, 2004; Sokol and Seksik, 2010) and that ROS dysregulation plays a critical role in the pathogenesis of these diseases (Bae et al., 2010; Grisham, 1994; Rokutan et al., 2008), the discovery of bacterial-derived uracil as an activator of gut-innate immunity and as a causal agent of colitogenesis will greatly help in better understanding the mucosal strategy of pathogen clearance versus commensal tolerance as well as the etiology of colitis associated with intestinal dysbiosis.

(F) Chronic activation of PLCβ-DUOX pathway by *G. morbifer*-derived uracil is the direct cause of host mortality. A log rank analysis on the Kaplan-Meier data showed a significant difference in survival between *G. morbifer* monoassociation and *G. morbifer-carA*::Tn5 monoassociation in the case of control flies ($p < 0.001$) but not in the case of *DUOX-RNAi* flies ($p = 0.59$) or *PLC*$\beta^{-/-}$ flies ($p = 0.47$).

Data were analyzed using an ANOVA followed by Tamhane's T2 post hoc test (A, C, and D) or by the Tukey post hoc test (B); values represent mean ±SEM (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$). See also Figures S6 and S7.

EXPERIMENTAL PROCEDURES

Fly Strains and Rearing

The fly lines used in this study are described in Extended Experimental Procedures. Flies were maintained at 25°C. The composition of standard corn meal agar medium was described previously (Shin et al., 2011). Axenic standard corn meal agar media were used for the experiments with germ-free animals or germ-free animals monoassociated with a commensal bacterium. Bokinin and propionic acid were omitted from the axenic media.

Bacterial Strains and Culture Conditions

E. carotovora subsp. *carotovora-15* was obtained from Bruno Lemaitre (Buchon et al., 2009b). *Commensalibacter intestini* A911^T, *Gluconobacter morbifer* G707^T, *Acetobacter pomorum*, *Lactobacillus plantarum*, and *Lactobacillus brevis* were isolated from our laboratory fly stocks (Roh et al., 2008; Ryu et al., 2008). Human-isolated bacterial strains were obtained from the Korean Collection for Type Cultures. We generated two URA$^-$ mutant bacteria, *E. carotovora-pyrE::*Tn*5* and *G. morbifer-carA::*Tn*5*, by using Tn5-mediated random mutant libraries for *E. carotovora* and *G. morbifer*. Detailed methods for Tn5-mediated random mutagenesis and screening strategy to isolate the URA$^-$ mutant are described in Extended Experimental Procedures and shown in Figures S3 and S6. *E. carotovora-pyrE::Tn5-pyrE* strain was generated by introducing *pTac3-pyrE* into *E. carotovora-pyrE::*Tn*5* strain. The *pTac3* expression vector containing apramycin resistance gene is a modified version of *pTac1* vector (Koo et al., 2003). All bacteria were cultured at 30°C. Appropriate antibiotics were added at 50 μg/ml (apramycin) and 30 μg/ml (kanamycin).

In Vivo ROS Detection in Intestinal Epithelia

R19S, a recently developed HOCl-specific rhodamine-based dye, was used in this experiment (Chen et al., 2011). The detailed methods used to measure bacterial-induced ROS and basal ROS levels are described in Extended Experimental Procedures.

Statistical Analysis

Comparisons of two samples were made by using either the Student's t test or the Mann-Whitney *U* test. Comparisons of multiple samples were made by ANOVA. The Kaplan-Meier log-rank test was used for the statistical analysis of fly survival experiments. p values of less than 0.05 were considered statistically significant. SPSS software (Chicago, IL, USA) was used for all analyses.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.04.009.

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